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Tuftsin–phosphorylcholine (TPC) equally effective to methylprednisolone in ameliorating lupus nephritis in a mice model

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Summary

The role of helminth treatment in autoimmune diseases is growing constantly. Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease with challenging treatment options. Tuftsin–phosphorylcholine (TPC) is a novel helminth-based compound that modulates the host immune network. This study was conducted to evaluate the potential value of TPC in ameliorating lupus nephritis in a murine model and specifically to compare the efficacy of TPC to the existing first-line therapy for SLE: corticosteroids (methylprednisolone). Lupus-prone NZBxW/F_1mice were treated with TPC (5 μg/mouse), methylprednisolone (MP; 5 mg/body weight) or phosphate-buffered saline (PBS) (control) three times per week once glomerulonephritis, defined as proteinuria of grade > 100 mg/dl, was established. Levels of anti-dsDNA autoantibodies were evaluated by enzyme-linked immunosorbent assay (ELISA), splenic cytokines were measured *in vitro* and the kidney microscopy was analysed following staining. TPC and MP treatments improved lupus nephritis significantly and prolonged survival in NZBxW/F_1 mice. TPC-treated mice showed a significantly decreased level of proteinuria ($P < 0{\cdot}001$) and anti-dsDNA antibodies ($P < 0{\cdot}001$) compared to PBS-treated mice. Moreover, TPC and MP inhibited the production of the proinflammatory cytokines interferon IFN-γ, interleukin IL-1β and IL-6 ($P < 0{\cdot}001$) and enhanced expression of the anti-inflammatory cytokine IL-10 ($P < 0{\cdot}001$). Finally, microscopy analysis of the kidneys demonstrated that TPC-treated mice maintained normal structure equally to MP-treated mice. These data indicate that the small molecule named TPC hinders lupus development in genetically lupus-prone mice equally to methylprednisolone in most of the cases. Hence, TCP may be employed as a therapeutic potential for lupus nephritis.

Keywords: helminths, lupus, phosphorylcholine, tuftsin-phosphorylcholine (TPC)

Introduction

Studies have shown that in regions where parasitic infections are endemic autoimmune diseases are rarely detected. Moreover, offspring of immigrants who move from an area of low-incidence of autoimmune diseases to one of high-incidence are likely to develop these diseases with a high incidence [1–3].

Helminths, a type of parasitic worm that can live symbiotically within humans, have been shown to influence the host immune system through several mechanisms. Subsequently, helminths and their ova have been employed in murine and human models of several autoimmune diseases, such as inflammatory bowel disease (IBD), type 1

diabetes mellitus (T1DM) and multiple sclerosis (MS). Improvement in common clinical disease activity indices has been demonstrated with no evidence of serious side effects or complications [4–8].

Secretion of immunomodulatory molecules is one mechanism by which helminths regulate our immune system. One such molecule, phosphorylcholine (PC) moiety, is presented by a specific secreted glycoprotein through N-glycans. PC is a non-immunogenic, immunomodulatory zwitterion found in Gram-negative bacteria, helminth-secreted molecules and on human cell membranes. It has been found that PC is a leading compound that modulates the host immune response [9,10].

In 1970, Najjar and Nishioka were the first to define tuftsin, an endogenous tetrapeptide (Thr-Lys-Pro-Arg) released during enzymatic cleavage of the Fc heavy chain of immunoglobulin IgG in the spleen. It can be recognized by receptors on monocytes and macrophages and has immunomodulatory properties [11,12]. Accordingly, we conjugated non-immunogenic PC with the self-natural immunomodulatory adjuvant, tuftsin, and created tuftsin–phosphorylcholine (TPC).

Systemic lupus erythematosus (SLE) is an autoimmune disease predominantly affecting young women by damaging the kidneys, brain, skin and nearly every organ in the body. Genetic, environmental, epigenetic, hormonal and immune regulatory factors influence the immune system in this multi-factorial disease. Renal disease (lupus nephritis) is considered the most severe manifestation of SLE, with an increased risk of morbidity and mortality [13–15].

Traditionally, SLE was treated with various anti-inflammatory drug families, including non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, anti-malarials and other immunosuppressants. In 2011, belimumab, a human monoclonal antibody against soluble B-lymphocyte stimulator (BLyS), was the first treatment for SLE to be approved by the Food and Drug Administration (FDA) in more than 50 years. However, each of these treatment options may cause a variety of adverse effects [13,14,16,17].

Accordingly, there is a need for novel therapeutic alternative for SLE patients with a less severe side effect profile. Our understanding of the pathogenesis of SLE and the immunomodulatory properties of helminth products has led us to study the effect of the aforementioned TPC compound on SLE.

We have shown previously that TPC induces a reduction in the activity indices in murine models. This includes down-regulation of proinflammatory cytokines, enhancement of the secretion of anti-inflammatory cytokines and expansion of the anti-inflammatory regulatory T cell (T_{reg}) phenotype [18–20].

In this study, we aim to assess the therapeutic potential of TPC in comparison to corticosteroids, which are the mainstay of SLE therapy, in an established experimental lupus murine model.

Materials and methods

TPC synthesis

Tuftsin (GLS peptide synthesis, Shanghai, China) was coupled to aminophenyphosphorylcholine (PC) (Biosearch Technologies, Inc. Novato, CA, USA), by Prof. Mati Fridkin (Department of Organic Chemistry at Weizmann Institute of Science, Rehovot, Israel). The peptide was coupled to diazotized 4-aminophenyphosphorylchloride to form an azo bond between the tuftsin and PC [19–21]. The conjugate was characterized by mass spectroscopy and amino acid analysis as well as by high-performance liquid chromatography (HPLC). The TPC was diluted in a commercial phosphate-buffered saline (PBS) (Biological Industries Israel Beit-Haemek Ltd, Kibbutz Beit-Haemek, Israel).

Mice and experimental design model

Female lupus-prone NZBxW/F_1 mice aged 12–13 weeks were purchased from Envigo (Alconbury, UK). The mice were maintained in a conventional animal housing facility at Sheba Medical Center, Israel, and kept in individually ventilated cages. All experiments were approved and executed according to the protocols of the Ethical Committee of the Israeli Ministry of Health no. 10056/16. The mice were assigned randomly to different treatment groups, each with 10 animals: TPC (5 μg/mouse subcutaneously), methylprednisolone (MP) sodium-succinate (5 mg/body weight; Pfizer, Puurs, Belgium) or PBS (control). The clinical course of renal involvement was monitored by assessing the levels of proteinuria, and the treatment was initiated after proteinuria (100 mg/dl) was observed at weeks 22–24. The treatment was administered three times per week.

Measurement of proteinuria

The urine was tested every week for proteinuria by a standard semi-quantitative Bayer Multistix (Bayer, Fernwald, Germany). The results were graded according to the manufacturer's instructions. Kidney damage was defined semi-quantitatively as the presence of 100 mg/dl of proteinuria.

Analysis of serum anti-dsDNA antibodies

Titres of anti-dsDNA antibodies were measured by enzyme-linked immunosorbent assay (ELISA), as described previously [22,23].

Cytokine measurements

Spleen cells were harvested from the mice. The spleen cells were depleted of erythrocytes by lysine (Biological Industries Israel Beit-Haemek Ltd). The splenocytes were seeded (5×10^5 cells/well) in 24-well plates (Thermo

plates; Nunc Fisher Scientific Inc., Waltham, MA, USA), coated with anti-CD3 antibodies (2 μg/ml) in the presence of 5 μg/ml TPC, MP or PBS for 72 h in RPMI-1640-enriched medium at 37°C and 5% CO_2 (Biological Industries Israel Beit-Haemek Ltd). Culture supernatants were then collected. Proinflammatory [interferon IFN-γ, interleukin IL-1β, IL-6] and anti-inflammatory IL-10 cytokine levels in the culture supernatant were detected by DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Histopathological analysis

Kidneys were obtained from mice killed at week 43 and were paraffin-embedded. The pathology of glomerulonephritis was exemplified by periodic acid-Schiff (PAS) histological staining. All microscopic evaluations were performed by a pathologist [19,20].

Statistical analysis

Data are expressed as mean ± standard deviation (s.d.). An analysis of variance (ANOVA) model using Dunnett's method (for adjustment to multiple comparisons) was applied to test the differences in anti-dsDNA antibody levels and cytokine profiles (IFN-γ, IL-1β, IL-6 and IL-10) between the three treatment groups as well as between each of the treatment groups compared to the reference group. Fisher's exact test was used to test statistically significant differences in the percentage of mice with urine protein levels greater than 300 mg/dl between the treatment groups. The log-rank test using Sidak's method was applied to analyse survival. *P*-values of less than 0·05 were considered to be statistically significant. The data were analysed using SAS® version 9.3 (SAS Institute, Cary, NC, USA).

Results

Level of anti-dsDNA antibodies titres in sera

The data presented in Fig. 1 describe anti-dsDNA antibodies titres in the sera of NZBxW/F_1 mice aged 37 weeks. The prevalence of serum anti-dsDNA antibodies was significantly lower in TPC-treated and MP-treated mice than in PBS-treated mice ($P < 0{\cdot}001$). In addition, the MP group had statistically significant reduced levels of anti-dsDNA antibodies compared to TPC treatment ($P < 0{\cdot}001$)

TPC and MP ameliorate kidney damage

We evaluated the clinical course of renal involvement in all three groups. Levels of proteinuria differed during the study period between treatment groups. As depicted in Fig. 2, while major proteinuria (urine protein ≥ 2000 mg/dl) was detected in 100% of PBS-treated mice at week 37, no mice from either the TPC- or MP-injected groups had reached this level of proteinuria ($P < 0{\cdot}001$). Regarding the level of proteinuria, treatment with TPC was non-inferior to treatment with MP ($P > 0{\cdot}1$).

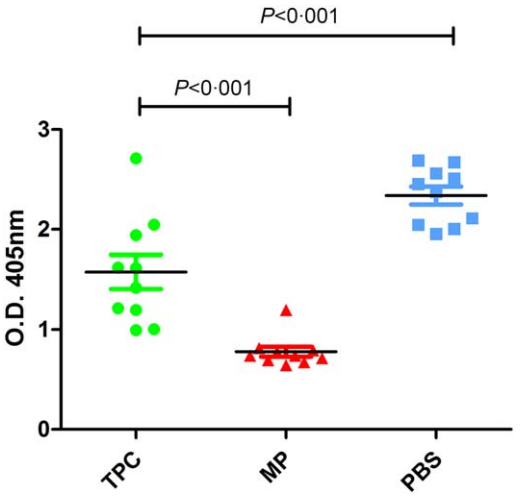

Fig. 1. Effects of tuftsin–phosphorylcholine (TPC) and methylprednisolone (MP) on serum levels of anti-dsDNA antibodies in a murine lupus model. Levels of circulating anti-dsDNA antibodies in the sera of NZBxW/F_1 mice treated with TPC, MP or phosphate-buffered saline (PBS) (control). Data were measured at week 37 by enzyme-linked immunosorbent assay (ELISA) at a dilution of 1 : 800 ($n = 10$ per group) and presented optical density (OD) at 405 nm.

Kidney morphology was evaluated at the end of the experiment. Kidneys from TPC-treated (Fig. 3a) and MP-treated mice (Fig. 3b) demonstrated the same morphological features of preserved renal parenchyma without signs of active glomerular disease. The glomeruli were observed with normal cellularity and without signs of thickening of

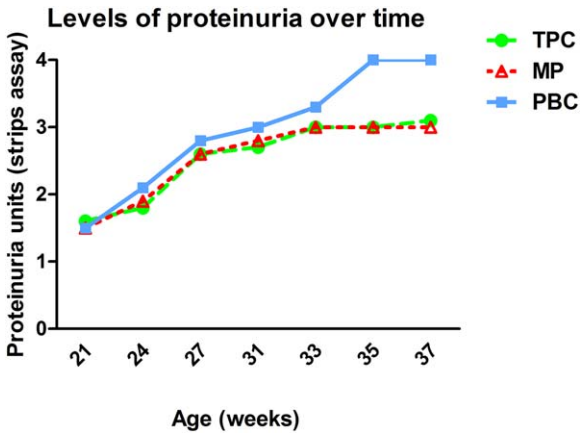

Fig. 2. Tuftsin–phosphorylcholine (TPC) and methylprednisolone (MP) ameliorated established lupus renal manifestations in a murine model. Changes in levels of proteinuria are presented in TPC, MP and PBS (control)-treated NZBxW/F_1 mice at various time-points during the study ($n = 10$ per group). $^*P < 0{\cdot}001$.

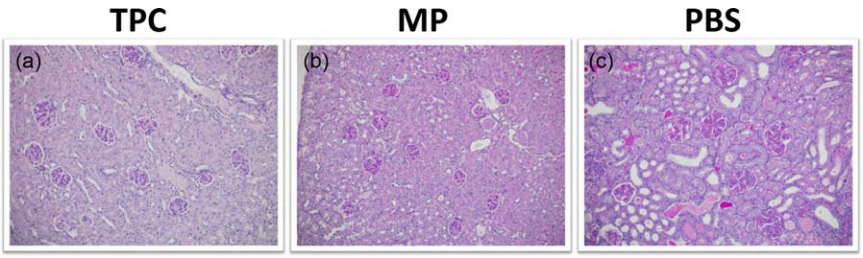

Fig. 3. Tuftsin–phosphorylcholine (TPC) and methylprednisolone (MP) eliminated glomerular and tubular damage in a murine lupus model. Representative slides from kidney sections of 37-week-old NZBxW/F_1 mice treated as indicated and stained with periodic acid-Schiff (PAS) from TPC, MP or PBS-treated mice ($n = 10$ per group). Original magnifications × 200. Photomicrographs from TPC (a) and MP (b) groups showed the same morphological features of minimal mesangial glomerulonephritis, class I (normal glomeruli). However, in PBS-treated mice (control) active proliferative diffused glomerulonephritis stage IV and damaged epithelial cells in the tubules are demonstrated in kidney histology (c).

glomerular capillary walls, correlating with class I (normal glomeruli).

In contrast, the renal parenchyma of PBS-treated mice showed proliferative diffuse glomerulonephritis class IV. This is represented by lobulated contours of glomerular tufts and proliferation of mesangial and endocapillary cells. Tubules displayed signs of focal damage to epithelial cells (Fig. 3c).

TPC and MP modulate cytokine profiles

TPC modulates the production of both pro- and anti-inflammatory cytokine production [18–20]. The mice treated with either TPC or MP promoted an anti-inflammatory environment, as exemplified by a 63- and sevenfold increase in IL-10 secretion levels, respectively, compared to PBS-treated mice ($P < 0{\cdot}001$). Moreover, a reduction in all proinflammatory cytokines was noted. Mice treated with TPC or MP had up to 13-fold lower levels of IL-1β, IL-6 and IFN-γ compared to the control group ($P < 0{\cdot}001$; Fig. 4). No statistical difference was detected between TPC and MP treatment groups ($P > 0{\cdot}1$).

TPC and methylprednisolone extend survival

It has been reported widely that *in-vitro* measurements do not always correlate with the prolongation of life. Thus, we evaluated the survival times for all three groups (Fig. 5). At week 41 of age, 71% of the PBS-treated mice had died. However, only 14 and 0% of the mice had died in the TPC and MP groups, respectively. Statistical significance was not attained between survival rates of TPC-treated mice and MP-treated mice ($P > 0{\cdot}3$).

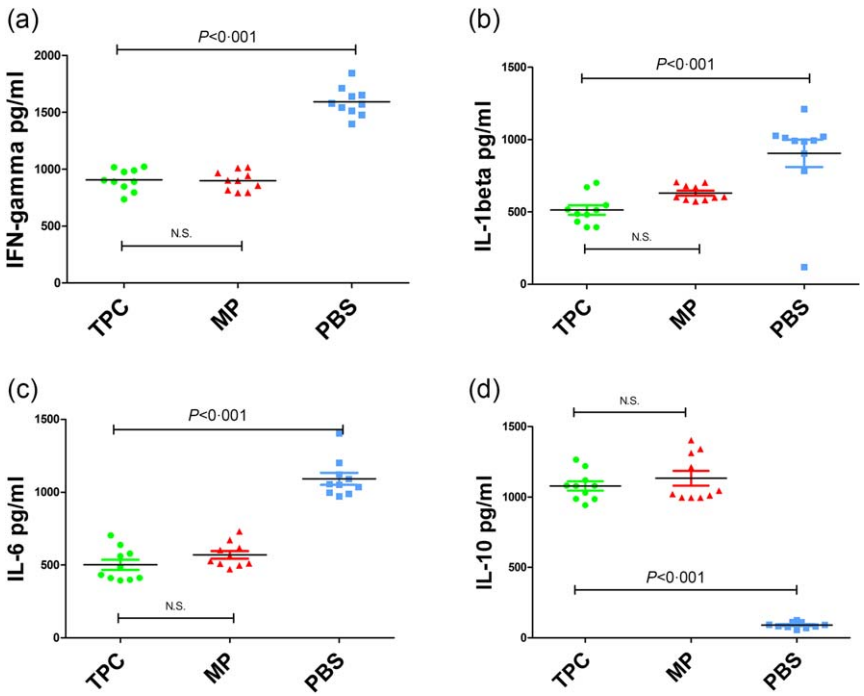

Fig. 4. Tuftsin-phosphorylcholine (TPC) and methylprednisolone (MP) modulate the expression of pro-inflammatory and anti-inflammatory cytokine in murine lupus model. *In-vitro* analyses of the pro-inflammatory cytokines IFN-γ (a), IL-1β (b), IL-6 (c) and anti-inflammatory cytokine IL-10 (d) cytokine concentrations in the culture fluids of splenocytes originated from TPC, MP and PBS (control) treated NZBxW/F1 mice. N.S. = non-significant.

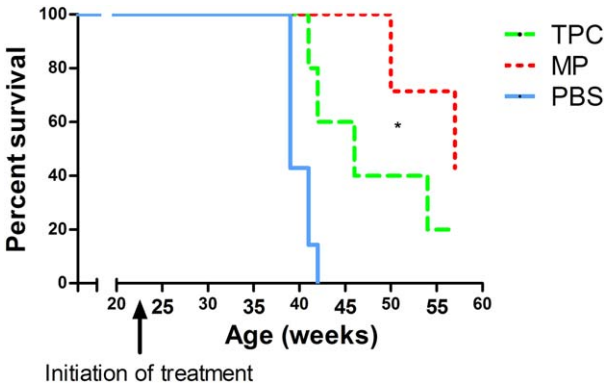

Fig. 5. Tuftsin–phosphorylcholine (TPC) and methylprednisolone (MP) prolonged survival in murine lupus model. Survival rates of TPC, MP and phosphate-buffered saline (PBS) (control)-treated NZBxW/F_1 mice ($n = 7$ per group). Data presented are expressed as percentage of mice that survived per week. *$P < 0{\cdot}001$, log-rank test.

Discussion

In the present study, we evaluated the therapeutic effects of TPC treatment compared to the standard first-line therapy for SLE – corticosteroids (MP) – and to a placebo (PBS) in a murine model.

Our results revealed that treatment of lupus mice with TPC attenuated kidney damage, as demonstrated by a low level of proteinuria ($P < 0{\cdot}001$), and preserved glomerular histology in a microscopic analysis. In fact, TPC-treated mice revealed similar kidney morphologies to mice treated with standard therapy for SLE (MP). Furthermore, in both treatment groups the proinflammatory cytokine profile was lowered significantly and the anti-inflammatory cytokine profile was elevated significantly ($P < 0{\cdot}001$). Finally, we showed that there was no significant difference in the extended survival within the TPC and MP treatment groups ($P > 0{\cdot}3$). Our results are in accordance with our previous studies of murine models treated with TPC: prevention of the development of lupus; reduction of disease activity score of dextransulphate sodium salt (DSS)-induced colitis; and attenuation of collagen-induced arthritis [18–20].

Glucocorticoid administration is by far the mainstay of treatment for SLE, especially at the onset of symptoms. Glucocorticoid treatment demonstrates widespread anti-inflammatory and immunosuppressive effects through direct and indirect transcriptional effects on the glucocorticoid receptor (GR). In particular, GR is responsible for the repression of the transcription factors nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) throughout myeloid and lymphoid cell types [24,25]. Therefore, we decided to explore further and compare the beneficial effect of TPC to that of glucocorticoids in the treatment of lupus. Although the exact cellular mechanism is still unknown, it has been well established that steroid use induces catabolism of antibodies, such as ds-DNA antibodies [26,27]. As illustrated in Fig. 1, MP-treated mice maintained a reduced level of anti-dsDNA antibodies, three times lower than PBS-treated mice and two times lower than TPC-treated mice.

While the mechanism of action of TPC has not yet been elucidated fully, tuftsin or tuftsin-like peptides have been shown to exert immune-stimulatory effects such as phagocytosis and cell migration [11,28–30]. In addition, it has been well established that helminth infections induce an immune response that shifts the host system towards T helper type 2 (Th2)-like phenotype. PC has also been shown to exert direct effects on the immune system through activation of dendritic cells via a variety of different Toll-like receptors (TLRs). Hence, we expect that treatment with both TPC and MP might have a synergistic effect due to the fact that the mechanism of action of both compounds is different [31–34].

Treatment of lupus nephritis relies strongly upon glucocorticoids [either methylprednisolone intravenously (i.v.) or orally], along with other immunosuppressants such as cyclophosphamide and mycophenolate mofetil. Recently, the FDA approved the first biological treatment with anti-BLYS for SLE patients, expanding the arsenal of therapies for this condition. However, long-term usage of immunosuppressive therapy poses the risk of numerous adverse effects. Moreover, not all patients respond to the first-line therapy and up to 44% of them fail to achieve long-term remission. Specifically, a higher incidence of malignancies and hypersensitivity reactions were reported in BLyS trials; investigation of long-term effects of biologics such as anti-BLYS is necessary. Treatment with other biological agents failed to inhibit lupus nephritis development [16,35–38].

Accordingly, there is an ongoing pursuit for a new and safe treatment for SLE. Over the years, *in-vitro* and *in-vivo* studies in experimental autoimmune models have proved that treatment with helminths, their ova or their extracts can influence the immune system and thereby ameliorate the clinical manifestations of several autoimmune diseases [4–8]. TPC was synthesized to create a new small molecule that would modulate the immune response. The approach we have taken in harnessing the immunomodulatory molecules found in helminths and their ova, together with that of self-peptide, could be a breakthrough in the field of autoimmune treatments [11,12,39,40].

This prospective, experimental laboratory animal study strengthens our assertion that TPC has an equal or better therapeutic effect on lupus nephritis than existing, albeit problematic, therapy. This is strengthened further by the fact that most outcomes were evaluated by either semi-quantitative or quantitative measurements, effectively evading or minimalizing observer bias.

However, our trial had several limitations. First, we did not compare TPC to other treatments for lupus nephritis, such as cyclophosphamide, mycophenolate mofetil or anti-

BLYS. Moreover, as this is a murine model, adverse reactions of TPC were not evaluated in comparison to methylprednisolone.

In summary, this study highlighted that TPC can ameliorate lupus nephritis in a murine model as effectively as methylprednisolone. Therefore, we believe that the small molecule of TPC could be of therapeutic value in the treatment of lupus nephritis.

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Disclosure

The authors declare no conflicts of interest.

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